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YAP enhances the pro-proliferative transcriptional activity of mutant p53 proteins

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1st Editorial Decision 07 May 2015

Thank you for your submission to EMBO reports. We have now received reports from the three referees that were asked to evaluate your study, which can be found at the end of this email. As you will see, although referee 3 is more positive, referees 1 and 2 appreciate the interest in the topic but consider that the data provided does not support the conclusions drawn or a role for YAP-mediated mutp53 activity in the context of human cancer.

As the reports are below, I will not detail them here. However, it is clear that substantial additional experimentation would be necessary to address the concerns regarding the conclusiveness, technical quality and physiological relevance of the results. In particular, strengthening the genetic data, extending the analysis to other p53 mutants, strengthening the molecular interaction data and the inhibitor-based studies, addressing potential discrepancies with your previous work, and addressing the concerns regarding technical quality (only one siRNA used, need for rescue experiments, small effects observed) would be necessary. All issues raised seem pertinent and are largely overlapping, and should thus be addressed.

Please note that it is our policy to undergo one round of revision only and thus, acceptance of your

study will depend on the outcome of the next, final round of peer-review.

Revised manuscripts must be submitted within three months of a request for revision unless previously discussed with the editor; they will otherwise be treated as new submissions.

When submitting your revised manuscript, we will require:

- a complete author checklist, which you can download from our author guidelines (http://embor.embopress.org/authorguide#revision)
- a letter detailing your responses to the referee comments in Word format (.doc)
- a Microsoft Word file (.doc) of the revised manuscript text
- editable TIFF or EPS-formatted figure files in high resolution
- a separate PDF file of any Supplementary information (in its final format)

In addition, EMBO reports will now start accommodating the inclusion of extra figures (up to five) in the online version of the manuscript. These are presented in an expandable format inline in the main text so that readers who are interested can access them directly as they read the article. They are also provided for download in a separate typeset PDF to accompany the Article PDF. These should be those of particular value to specialist readers, but which are not required to follow the main thread of the paper (and not additional controls or reagent optimization). These should be labeled expanded view, and the rest supplementary.

We also welcome the submission of cover suggestions or motifs that might be used by our Graphics Illustrator in designing a cover.

I look forward to seeing a revised form of your manuscript when it is ready. In the meantime, please contact me if I can be of any assistance. Should you feel
that the revision required is too extensive and choose to submit your paper elsewhere, please let me know.

REFEREE REPORTS

Referee #1:

In this study, the authors reported that YAP interacts with mutant p53 to bind the NFY transcription to induce cell cycle genes, therefore to stimulate cell proliferation. Based on bioinformatic analysis, the authors observed that mutant p53 and YAP share common set of genes, particularly those involved in cell cycle. Using luciferase reporter or endogenous mRNA, YAP and mutant p53 were found to be co-dependent in regulating cyclin B. Moreover, co-immunoprecipitation showed that YAP and p53 interacted with each other and formed a complex with NFY. The authors conclude that mutant p53, YAP, and NFY form a functional complex to bind the CCATT box to stimulate gene expression, particularly cell cycle genes. YAP is well documented to promote tissue and organ growth while mutant p53 often accumulates in cancers and may have a positive role in tumorigenesis. This study proposes a rather provocative and potentially model regarding the function of mutant p53 and YAP in tumorigenesis.

Comments

However, there is no genetic evidence suggesting a functional interdependence between mutant p53 and YAP. In fact, the existing genetic data argue against such a model. For example, genetic studies using mouse models or other organisms have shown that YAP activation, either by overexpression or mutation of the upstream negative regulators, can strongly stimulate cell proliferation, organ size, and tumorigenesis. Therefore, mutant p53 is not required for YAP to stimulate cell growth. Moreover, studies in cell culture also showed that YAP activation promotes cell growth in p53 wild type (such as MEF) cells. Finally, there is no genetic data indicating that YAP is elevated in p53 mutant cancers. Therefore, the observations described in the current study may not be important to cell growth regulation by mutant p53 and YAP, hence the relevance to cancer is unclear.

Does YAP and mutant p53 affect each other's expression in their respective knockdown experiments

(Fig 1C, 1D)?

Based on the analyses in Fig 2A, 2B, expression of YAP signature genes also depends on the wt-p53 (the right two columns of each panel). Does this mean that wt-53 also activates YAP signature gene expression and how? Because YAP is an oncogene, one would expect that patients with high YAP have a poor survival (Fig 2C). This may have nothing to do with the p53 status. What is the survival curve of wt-p53 patients with high or low YAP?

The key observation of this study is that mutant p53, but not the wtp53, interacts with YAP. This is very surprising and interesting. There are a large number of p53 point mutants that have been characterized regarding their role in cancer. The authors should test a large panel of p53 mutants to show whether their ability to interact with YAP correlates their role in cancer to strengthen the key point of this study. If many mutant p53 can interact with YAP, can the authors explain how single amino acid substitutions on many different places on the p53 protein could gain a common function, interacting with YAP? Moreover, the authors should perform deletion mapping to define the region of mutant p53 responsible for interaction with YAP. Reciprocal immunoprecipitation with p53 antibody, and NFY antibody should be done in Fig 4C. Co-immunoprecipitation with each one of the three antibodies (YAP, p53, NFY) should be determined in cells with knockdown of p53 alone, YAP alone, and NFY alone to confirm the trimeric complex and their relationship.

Inhibition of the mevalonate pathway could have a very broad effect, such as blocking isoprenylation of Ras (Fig 6). Additional experiments, such as reversion of the inhibitor effect by YAP overexpression, are need to demonstrate these effects exerted by the inhibitors are indeed due to YAP inhibition. In addition, YAP nuclear and cytoplasmic localization should be shown to confirm he effect of the inhibitors. What is the effect of cerivastatin on cell proliferation and expression of cell cycle genes in wt-p53 cells and p53 null cells (preferably isogenic cell lines)? Cyclin B is not a well-defined YAP target gene. Why did the authors use cyclin B, which is not a well-defined YAP target genes?

Referee #2:

In this manuscript "YAP enhances the pro-proliferative transcriptional activity of mutant p53 proteins" (EMBOR-2015-40488V1), authors has proposed an interesting model to explain the gain-of-function activities of mutant p53 that account for expression of cyclin A, B, CDK1 and increased cell proliferation. However, given that the evidence provided is still preliminary, the major conclusions still remain to be determined.

Specific Comments:
1. Authors' previous publication regarding mutant p53, CCAAT box and cyclin A, B, CDK1 was mainly based on the observation after treatment of Adriamycin (Cancer Cell, 10:191). Therefore, it is surprising that in current study mutp53 knockdown exhibited the impact on cyclin A, B, CDK1 expression even without DNA damaging agent treatment (Fig. 1C). The reason of this inconsistent observation is unclear. Since according to authors' original proposal, in the absence of DNA damaging agent Adriamycin, mutant p53/NF-Y complex is not supposed to bind to the promoters of the above-mentioned target genes to regulate gene expression (Cancer Cell, 10:191); Similarly, it is also surprising that in current study mutant p53 binds to the promoters of those genes in the absence of Adriamycin stimulation (Fig 4F-G);
2. All the experiments described in this study were solely based on a single siRNA for p53, YAP1 and NF-Y (Figures 1, 3, 4, 5, 6). Therefore, the accuracy of all results obtained need to be further validated; Moreover, YAP activity is usually associated with cell culture conditions (e.g. cell density and others), it raises the possibility that the difference of some results obtained may not be due to mutant p53;
3. The effect of YAP knockdown on expression of cyclin A, B, CDK1 is minimal given that the internal control GAPDH appeared to be also affected by YAP knockdown (Fig. 1D);
4. Neither p53 nor YAP knockdown was shown in SKBR3 cells (Fig. 3 A);
5. Almost all the IP experiments in this study used a single YAP antibody. However, the p53 pull-down band appeared to be larger than that in the extracts with a slower migration (e.g. Fig. 4A, 4C, S2D). Therefore, it is possible that YAP antibody pulled down non-specific band or it is YAP IgG cross-reaction (although IgG was used as control, it is unclear the amount and subtype of the IgG

used);
6. The reciprocal mutp53 IP using p53 antibody was only shown in Fig. 4B, S2A in an overexpression system. However, it is unclear whether the interaction came from GFP & mutp53 R175H interaction (Fig. 4B) or from the non-specific false cross-reactive band (since it migrated slower than the endogenous YAP1) (Fig. S2A)?
7. In Fig. 4E, Ig G pulled down approximately 1/3 of NF-YB (lane 2) when compared to NF-YB antibody (lane 3). If mutp53, YAP and NF-YB truly stay in the same complex as authors suggested, it is hard to believe that none of YAP and p53 were detected in IgG lane (lane 2) [it should have been 1/3 of that in NF-YB lane (lane 3), right?] (Fig. 4E).
Taken together, the evidence of mutp53 and YAP interaction provided thus far is not supportive for the hypothesis;
8. Given the importance of the oncogenic YAP1, experiments in Fig. 5A-D with siYAP did not provide any useful information to support authors' hypothesis; In addition, siRNA effect in Fig. 5E is questionable. Since si-p53 in Fig. S4D did not show any effect on p53 expression (compare lane 4 to lane 1, Fig. S4D). In addition, if authors' hypothesis is correct that levels of YAP and mutp53 could determine the cell proliferation, the proliferation rate shown in Fig. 5E should have been YAP >YAP+sip53 >siGFP >sip53, because this is the exact order of YAP & mutp53 expression levels as shown in Fig. S4D, in which YAP & mutp53 expression levels are: YAP (lane 3)>YAP+sip53 (lane 4) >siGFP (lane 1, Fig. S4D)>sip53 (lane 2).
9. The specificity of drugs cerivastin and ZA is questionable. e. g. they may affect cyclin A, B, CDK1 and even p53 expression directly, which may has nothing to do with YAP, or vice versa.

Referee #3:

The manuscript by di Agostino et al. outlines the novel finding that mutant p53 protein promotes increased oncogenic behaviour through YAP mediated activation of NF-Y on cell cycle regulated genes. This is study represents an innovative bioinformatic analysis that clearly points to overlap of both p53mut activity and YAP pathway in cancer. The work is exemplary and has appropriate controls and clear experimental reasoning, moreover this work provides crucial evidence for aggressiveness of p53mut cancers while also highlighting therapeutic angles. It is my feeling that work should be published with minimal delay.

The only criticism is that some discussion should be devoted to three points
1) Whether the authors have evidence that there is a natural role for YAP/NF-Y in transcription that is perturbed or altered by mutant p53, or is YAP/NF-Y a consequence of oncogenic transformation?

2) Mutant p53 interacts with both p63 and p73 inhibiting their respective activities, therefore are the findings reported here independent of alternative family members? For example is the p53mut-YAP interaction dependent on p63 or p73? Does p53mut adopt a p73 like structure that facilitates binding?

3) The authors do not address pS127-YAP1 or the nuclear localisation of YAP in this context. I would expect from their findings that pS127 is low and YAP is nuclear to allow this interaction. However, as p53mut protein is found at high levels in the cytoplasm, it equally feasible that there is both high levels of pS127-YAP in the cytoplasm that could equally be bound by p53mutant AND a level of YAP in the nucleus (p53mut bound) that remains transcriptionally active. This would be interesting follow on work but the authors should at least comment in the discussion.

1st Revision - authors' response 17 July 2015

We wish to thank you very much for your efforts in handling our manuscript (EMBOR-2015-40488V1) and for giving us the opportunity to revise and improve it. We greatly appreciated your thoughtful and constructive comments as those from the reviewers. We revised the manuscript accordingly.

As the reports are below, I will not detail them here. However, it is clear that substantial additional experimentation would be necessary to address the concerns regarding the conclusiveness, technical quality and physiological relevance of the results. In particular,

strengthening the genetic data, extending the analysis to other p53 mutants, strengthening the molecular interaction data and the inhibitor-based studies, addressing potential discrepancies with your previous work, and addressing the concerns regarding technical quality (only one siRNA used, need for rescue experiments, small effects observed) would be necessary. All issues raised seem pertinent and are largely overlapping, and should thus be addressed.

In particular we now provide the following evidences:

a) **strengthening the genetic data:** There is not yet full genetic evidence linking mutantp53 and YAP. However several convincing evidences point towards this link. Indeed, we have previously reported that YAP intercepts mutantp53- mediated metabolic signalling pathways in cell culture systems (Sorrentino et al., 2014). Moreover, in the same study, by using TCGA datasets, we found a strong correlation between the presence of missense mutant p53 in breast cancer and YAP activity. Additional proof is provided by the recent findings of Zhang and colleagues (Sci Signal. 2014 May 6;7(324):ra42. doi: 10.1126/scisignal.2005049). They showed that YAP deletion ($Yap^{flox/flox}$ mice) in the $Kras^{G12D/+}$:$p53^{R172H/+}$ mice determined the arrest of the proliferation without affecting pancreatic development and endocrine function. In this revised version of the manuscript we now show that breast tumors (Metabric dataset) carrying p53 mutations and high expressed YAP-gene signature exhibit a lower survival when compared to both those carrying p53 gene mutations and low expressed YAP signature and with those carrying wt-p53 protein and high expressed YAP gene signature (new Figure 2C). In the new version of Figure 5C we also show that YAP enhanced cell proliferation of mutant p53 cell line such as CAL27. This effect was lost when the cells were depleted of mutant p53 protein expression (new version of Figure 5C and Supplementary Figure 5F).

Altogether these evidences provide with the strong rationale to investigate the molecular mechanisms underlying the mutp53/YAP interdependence and fill up the existing lack of related knowledge. Here we contribute to these aims by providing molecular evidence that YAP potentiates mutantp53 gain of function by tethering a transcriptional competent protein complexes including YAP, mutant p53 and the transcription factor NFY.

b) **extending the analysis to other p53 mutants:** To this end we have assessed the presence of the protein complex mutp53/YAP in additional tumour cell lines carrying mutant p53 proteins. In detail, we now show in the new Supplementary Figure 3B that mutp53/YAP protein complex is present in MDA-MB-231 breast cancer cells, FADU head and neck cancer cell line and PANC1 pancreatic cancer cells. We also performed these co-precipitation experiments with an additional anti-YAP antibody.

c) **strengthening the molecular interaction data:** To this end we have assessed the presence of a triple complex including mutp53, YAP and the transcription factor NFY. We now show in the new Figure 4F that a triple complex mutp53/YAP/NFY is present in MDA-MD-468 cells. We also show that mutp53/YAP complex is still present in cells depleted for NFY-B expression (Figure 4G). Since in NFY depleted cells we did not find any mutp53/YAP protein complex onto the promoters of cell cycle regulated genes (Figure 4I), all these findings indicate that NFY is not necessary for the formation of the mutp53/YAP complex but it is required for its recruitment on cell cycle promoters. A descriptive model is shown in Figure 4J.

We also show in the new Figure 4E that deletion of a region of mutant p53 protein comprised between aa 338 to aa 355 reduced its binding to YAP. This might suggest that structural alteration or post-translation modifications within this region impact on the formation of mutp53/YAP protein complex.

d) **strengthening the inhibitor-based studies:** To this aim we reintroduced the statin-insensitive (Sorrentino et al. 2014) YAP-5SA (siRNA resistant) in MDA-MB-231 depleted of endogenous YAP and checked the expression of cell cycle genes after statin treatment. The result is shown in new Supplementary Figure 6B and demonstrates that statin treatment fails to reduce the expression of cyclin A, cyclin B and Cdc25c in cell reconstituted with the statin-insensitive YAP mutant. In the same experimental set-up, viability assay shown in new Supplementary Figure 6C, demonstrates that Cerivastatin is significantly less efficient in reducing proliferation in YAP-5SA-expressing cells. These results prove that YAP nuclear activity plays a major role in mediating the inhibitory

effect of statins on cell cycle gene expression and cell proliferation.

e) **addressing potential discrepancies with your previous work:** To this aim we wish to evidence as reported in the Figure 4C in Di Agostino et al., 2006 that the presence of mutant p53 and NFY onto the CCAAT boxes of cyclin B and CDK1 promoters was already evident at the basal level. DNA damaging agents (Figure 4C and Figure 6A in Di Agostino et al., 2006) increased the recruitment of the protein complex mutant p53 and NFY onto cell cycle gene promoters. Additional evidence on this vein has been reported by Liu et al., 2011 (Liu et al., Mol Cell Biol. 2011 Nov;31(22):4464-81. doi: 10.1128/MCB.05574-11) and Acin et al., 2011 (Acin et al., J Pathol. 2011 Dec;225(4):479-89. doi: 10.1002/path.2971). Indeed Acin et al. reported that endogenous p53 GOF mutation $p53_{R172H}$, but not deletion of *p53*, cooperates with oncogenic K-ras during HNSCC initiation, accelerates oral tumour growth, and promotes progression to carcinoma. The expression profiling of the tumours that developed in these mice and studies using cell lines derived from these tumours determined that mutant p53 induces the expression of genes involved in mitosis, including cyclin B1 and cyclin A, and accelerates entry in mitosis. Accordingly with our findings reported in Di Agostino et al. 2006, doxorubicin treatment of explanted tumor cells increased further the expression of cyclin B1 and cyclin A in mutant p53 expressing cells.

f) **addressing the concerns regarding technical quality:** To this end we performed new sets of experiments by using additional siRNAs for both mutant p53 and YAP expression. In detail, the related data for both protein and mRNA expression of cell cycle regulated genes are shown in the new right panels C and D of the new version of Figure 1 and the new Supplementary Figure 1B-C and new Supplementary Figure 2 C-E. We also used additional anti-YAP antibody to perform the coimmunoprecipitation experiments shown in the new Supplementary Figure 3B.

Below please find the detailed, itemized list of our responses to the reviewers' suggestions/ comments and the changes we have made in the revised version of the manuscript.

Referee #1:
In this study, the authors reported that YAP interacts with mutant p53 to bind the NFY transcription to induce cell cycle genes, therefore to stimulate cell proliferation. Based on bioinformatic analysis, the authors observed that mutant p53 and YAP share common set of genes, particularly those involved in cell cycle. Using luciferase reporter or endogenous mRNA, YAP and mutant p53 were found to be co-dependent in regulating cyclin B. Moreover, co-immunoprecipitation showed that YAP and p53 interacted with each other and formed a complex with NFY. The authors conclude that mutant p53, YAP, and NFY form a functional complex to bind the CCATT box to stimulate gene expression, particularly cell cycle genes. YAP is well documented to promote tissue and organ growth while mutant p53 often accumulates in cancers and may have a positive role in tumorigenesis. This study proposes a rather provocative and potentially model regarding the function of mutant p53 and YAP in tumorigenesis.

Comments
However, there is no genetic evidence suggesting a functional interdependence between mutant p53 and YAP. In fact, the existing genetic data argue against such a model.

For example, genetic studies using mouse models or other organisms have shown that YAP activation, either by overexpression or mutation of the upstream negative regulators, can strongly stimulate cell proliferation, organ size, and tumorigenesis. Therefore, mutant p53 is not required for YAP to stimulate cell growth. Moreover, studies in cell culture also showed that YAP activation promotes cell growth in p53 wild type (such as MEF) cells.

Response: There is not yet full genetic evidence linking mutantp53 and YAP. However several convincing evidences point towards this link. Indeed, we have previously reported that YAP intercepts mutantp53-mediated metabolic signalling pathways in cell culture systems (Sorrentino et al., 2014). Moreover, in the same study, by using TCGA datasets, we found a strong correlation between the presence of missense mutant p53 in breast cancer and Yap activity.

Additional proof is provided by the recent findings of Zhang and colleagues (Sci Signal. 2014 May 6;7(324):ra42. doi:10.1126/scisignal.2005049). They showed that YAP deletion ($Yap_{flox/flox}$ mice) in

the *Kras*$^{G12D/+}$:*p53*$^{R172H/+}$ mice determined the arrest of the proliferation without affecting pancreatic development and endocrine function.

In this revised version of the manuscript we now show that breast tumors (Metabric dataset) carrying p53 mutations and high expressed YAP-gene signature exhibit a lower survival when compared to both those carrying p53 gene mutations and low expressed YAP signature and with those carrying wt-p53 protein and high expressed YAP gene signature (new Figure 2C). In the new version of Figures 5C-D we also show that YAP enhanced cell proliferation of mutant p53 cell lines such as MDA-MB-231and Cal27. This effect was lost when the cells were depleted of mutant p53 protein expression (new version of Figures 5C-D). Altogether these evidences provide with the strong rationale to investigate the molecular mechanisms underlying the mutp53/YAP interdependence and fill up the existing lack of related knowledge. Here we contribute to these aims by providing molecular evidence that YAP potentiates mutantp53 gain of function by tethering a transcriptional competent protein complexes including YAP, mutant p53 and the transcription factor NFY.

Finally, there is no genetic data indicating that YAP is elevated in p53 mutant cancers.Therefore, the observations described in the current study may not be important to cell growth regulation by mutant p53 and YAP, hence the relevance to cancer is unclear.

Response: YAP transcriptional activity is determined by its nuclear localization rather than its expression. Recently we have shown that the depletion of mutant p53 in MDA-MB-231 cells inhibits YAP/TAZ activity as judged by the reduction in nuclear localization of YAP/TAZ and reduced transcriptional activity (Fig. 6e§h and Supplementary Fig. 7b,c; Sorrentino et al., Nature Cell Biol 2014 DOI: 10.1038/ncb2936). In the same study we analyzed the YAP/TAZ activity in breast cancer patients (TCGA) using well established YAP/TAZ gene signatures (Zhang et al., 2009; Dupont et al., 2011). After patient stratification based on the presence of mutant p53, we found that samples expressing missense mutant p53 exhibited higher activation of YAP/TAZ.

In the present manuscript, by using the Metabric cohort, we now show (Figure 2C) that patients with missense mutant p53 and high YAP activity have a significant poor prognosis when compared to patients with wtp53 and high YAP activity. Altogether these evidences suggest that YAP/TAZ could be a relevant executor of the pro-oncogenic functions of mutant p53.

Does YAP and mutant p53 affect each other's expression in their respective knockdown experiments (Fig 1C, 1D)?

Response: We have preliminary observations (that we share with the reviewer) showing that YAP depletion did not affect mutant p53 expression while mutant p53 depletion slightly reduced YAP expression. This is an interesting issue that suggest another level of the interplay between mutant p53 and YAP that is in line with all our observations. However this goes beyond the scope of the present manuscript and will be addressed in future studies.

Based on the analyses in Fig 2A, 2B, expression of YAP signature genes also depends on the wt-p53 (the right two columns of each panel). Does this mean that wt-53 also activates YAP signature gene expression and how? Because YAP is an oncogene, one would expect that patients with high YAP have a poor survival (Fig 2C). This may have nothing to do with the p53 status. What is the survival curve of wt-p53 patients with high or low YAP?

Response: As already pointed above, we now show that breast tumours (Metabric dataset) carrying p53 missense mutations and high levels of YAP-gene signature have a lower survival when compared to patients carrying wt p53 and high levels of YAP signature (new Fig. 2C).

The key observation of this study is that mutant p53, but not the wtp53, interacts with YAP. This is very surprising and interesting. There are a large number of p53 point mutants that have been characterized regarding their role in cancer. The authors should test a large panel of p53 mutants to show whether their ability to interact with YAP correlates their role in cancer to strengthen the key point of this study.

Response: To address this concern we performed co-precipitation assays for the detection of the

protein complex mutp53/YAP in a panel of eight cancer cell lines expressing conformational (e.g. p53 H175) and contact defective (p53 H273) p53 mutants (MDA-MB468, SKBr3, SW480, T47D, CAL27) (Figure 4C) and FADU, MDA-MB-231 and PANC1 (Supplementary Figure 3C). These cancer cell lines are representative of breast, colon, head and neck and pancreatic tumors therefore the complex mutp53/YAP is not specific only for breast cancer.

We have also analyzed the expression of cell cycle related gene (mRNA and protein) in these additional cell lines upon knock-down of mutant p53 and YAP expression (new Supplementary Figure 1B-C and new Supplementary Figure 2CE). Transactivation assays using the wild type and mutated LUC-CCNB promoter was additionally performed in MDAMB-468 cells expressing mutp53R273H, a canonical DNA-contact defective p53 mutant (new Figure 3A).

If many mutant p53 can interact with YAP, can the authors explain how single amino acid substitutions on many different places on the p53 protein could gain a common function, interacting with YAP? Moreover, the authors should perform deletion mapping to define the region of mutant p53 responsible for interaction with YAP.

Reponse: Despite a single amino acid change the resulting mutant p53 proteins have common features. They loose wtp53 transcriptional activity and consequently its related tumor suppressor activities; their half-life is significantly prolonged and consequently they are abundantly present in cancer cells, they gain oncogenic function also through the interaction with transcription factors. The transcriptional cross-talk with diverse transcription factors such as E2F, NFkB, SP1, ETS1 (Fontemaggi et al., 2009 Nat Struct Mol Biol. 2009 doi: 10.1038/nsmb.1669; Weisz et al., 2007 Cancer Res. 2007 Mar 15;67(6):2396-401; Sampath et al., 2001 J Biol Chem 276: 39359-39367; Dell'Orso et al., 2011 OMICS doi: 10.1089/omi.2010.0084) occurs irrespectively from the type of p53 mutants. However it is conceivable that all these interactions could be modulated in part in a cell context dependent manner also by post-translational modifications, many of them occurring also in wtp53 (Nguyen et al., Human Mutation 2014, DOI:10.1002/humu.22506). In general however this is a field that still needs investigation. Nevertheless we attempted to define the contact region of mut p53 with YAP and in the new Figure 4E we now show that this could be comprised between p53 aa 338 to aa 355. Indeed deletion of this mutant p53 region reduced its binding to YAP suggesting that structural alteration or posttranslational modifications within this domain impact on the formation of mutp53/YAP protein complex.

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Reciprocal immunoprecipitation with p53 antibody, and NFY antibody should be done in Fig 4C
Response: We have performed reciprocal co-immunoprecipitation experiments in CAL27, MDA-MB-231, FADU and PANC1 cell lines using an additional antibody anti-YAP (goat polyclonal by Santa Cruz SC-1714). YAPimmunoprecipitates were probed with anti-YAP and anti-p53 antibodies

(new Supplementary Figure 3C). To further consolidate the findings related the existence of the YAP/mutp53/NFY protein complex, we have performed in MDA-MB-468 cells immunoprecipitations using anti-YAP, anti-p53 and anti-NFY antibodies. Each of the immunoprecipitates was probed with anti-YAP, anti-p53 and anti-NFY antibodies (new Figure 4F).

Co-immunoprecipitation with each one of the three antibodies (YAP, p53, NFY) should be determined in cells with knockdown of p53 alone, YAP alone, and NFY alone to confirm the trimeric complex and their relationship.

Response: In the Figure 4C we now show that the depletion of NF-Y did not impact on the formation of the mutp53/YAP protein complex. However, as shown in Figure 4I the NF-Y knock-down impaired severely the recruitment of mutp53/YAP complex onto the CCATT box sequences of the cell cycle related target gene promoters (Figures 4I and J).

Inhibition of the mevalonate pathway could have a very broad effect, such as blocking isoprenylation of Ras (Fig 6). Additional experiments, such as reversion of the inhibitor effect by YAP overexpression, are need to demonstrate these effects exerted by the inhibitors are indeed due to YAP inhibition.

Response: We agree with the referee's comments and we performed new experiments to address his point. As suggested by the referee, we formally prove that the inhibitory effect of Cerivastatin on the expression of cell cycle genes is mainly due to inhibition of nuclear YAP activity in mutant-p53 expressing cells. To this aim we reintroduced the statin-insensitive (Sorrentino et al. 2014) YAP-5SA (siRNA resistant) in MDA-MB-231 depleted of endogenous YAP and checked the expression of cell cyle genes after statin treatment. The result is shown in new Supplementary Figure 6B and demonstrates that statin treatment fails to reduce the expression of cyclin A, cyclin B and Cdc25c in cell reconstituted with the statin-insensitive YAP mutant. In the same experimental set-up, viability assay shown in new Supplementary figure 6C, demonstrates that Cerivastatin is significantly less efficient in reducing proliferation in YAP- 5SA-expressing cells. These results prove that YAP nuclear activity plays a major role in mediating the inhibitory effect of statins on cell cycle gene expression and cell proliferation.

In addition, YAP nuclear and cytoplasmic localization should be shown to confirm the effect of the inhibitors.

Response: Concerning this point, we have monitored the nuclear YAP localization in all the experiments performed with Cerivastatin. The new Supplementary Figure 6A shows a representative immunofluorescence of YAP subcellular localization as well as a quantification of YAP nuclear localization after Cerivastatin treatment in MDA-MB-231.

What is the effect of cerivastatin on cell proliferation and expression of cell cycle genes in wt-p53 cells and p53 null cells (preferably isogenic cell lines)?

Response: To address this point, we treated wild type p53-expressing breast cancer cell lines MCF7 with cerivastatin. As shown by new Supplementary Figure 6F, in this cellular context, Cerivastatin was completely unable to inhibit the expression of cell cycle genes and showed a very mild effect on cell proliferation (as already reported: Denoyelle et al. 2000; Supplementary Figure 6E). These effects were largely p53 independent, since p53 knock-down by siRNA transfection had any effect on cell cycle gene expression and cell proliferation upon Cerivastatin treatment.

Referee #2:
In this manuscript "YAP enhances the pro-proliferative transcriptional activity of mutant p53 proteins" (EMBOR-2015-40488V1), authors has proposed an interesting model to explain the gain-of-function activities of mutant p53 that account for expression of cyclin A, B, CDK1 and increased cell proliferation. However, given that the evidence provided is still preliminary, the major conclusions still remain to be determined.

Specific Comments:
1. Authors' previous publication regarding mutant p53, CCAAT box and cyclin A, B, CDK1 was mainly based on the observation after treatment of Adriamycin (Cancer Cell, 10:191). Therefore, it is surprising that in current study mutp53 knockdown exhibited the impact on cyclin A, B, CDK1 expression even without DNA damaging agent treatment (Fig. 1C). The reason of this inconsistent observation is unclear. Since according to authors' original proposal, in the absence of DNA damaging agent Adriamycin, mutant p53/NF-Y complex is not supposed to bind to the promoters of the above-mentioned target genes to regulate gene expression (Cancer Cell, 10:191); Similarly, it is also surprising that in current study mutant p53 binds to the promoters of those genes in the absence of Adriamycin stimulation (Fig 4F-G).

Response: We wish to thank the reviewer for his/her comment which provide with the possibility to clarify the related issues. We wish to evidence as reported in the Figure 4C in Di Agostino et al., 2006 that the presence of mutant p53 and NFY onto the CCAAT boxes of cyclin B and CDK1 promoters was already evident at the basal level. DNA damaging agents (Figure 4C and Figure 6A in Di Agostino et al., 2006) increased the recruitment of the protein complex mutant p53 and NFY onto cell cycle gene promoters. This finding is also evident in the Figure 6A (Di Agostino et al., 2006) in which a basal recruitment (in the absence of DNA damage treatment) of mutant p53 and NF-Y onto the ectopic promoter region (promCCAAT-B2 LUC) was already evident and further increased after 24h of ADR treatment. Additional evidence has been reported byby Liu et al., 2011 (Liu et al., Mol Cell Biol. 2011 Nov;31(22):4464-81. doi: 10.1128/MCB.05574-11) and Acin et al., 2011 (Acin et al., J Pathol. 2011 Dec;225(4):479-89. doi: 10.1002/path.2971).

Indeed, Acin et al reported that endogenous p53 GOF mutation p53R172H, but not deletion of *p53*, cooperates with oncogenic K-ras during HNSCC initiation, accelerates oral tumour growth, and promotes progression to carcinoma. The expression profiles of the tumours that developed in these mice and studies using cell lines derived from these tumours determined that mutant p53 induced the expression of genes involved in mitosis, including cyclin B1 and cyclin A, and accelerates entry in mitosis. In agreement with our findings reported in Di Agostino et al 2006, doxorubicin treatment of explanted tumor cells increased further the expression of cyclin B1 and cyclin A in mutant p53 expressing cells.

2. *All the experiments described in this study were solely based on a single siRNA for p53, YAP1 and NF-Y. Therefore, the accuracy of all results obtained need to be further validated;*

Response: To this end we performed new sets of experiments by using additional siRNAs for both mutant p53 and YAP expression. In detail, the related data for both protein and mRNA expression of cell cycle regulated genes are shown in the new right panels C and D of the new version of Figure 1 and the new Supplementary Figure 1B-C and new Supplementary Figure 2 C-E. We also used additional anti-YAP antibody to perform the coimmunoprecipitation experiments shown in the new Supplementary Figure 3B.

Moreover, YAP activity is usually associated with cell culture conditions (e.g. cell density and others), it raises the possibility that the difference of some results obtained may not be due to mutant p53.

Response: We wish to thank the reviewer for pointing out this issue. All the experiments were performed with cells cultured at 50-60% confluence to avoid any impact of cell density on the presented findings. A representative example of the cell confluence culture condition in which YAP is clearly nucler is shown in the new Supplementary Figure 6A.

3. The effect of YAP knockdown on expression of cyclin A, B, CDK1 is minimal given that the internal control GAPDH appeared to be also affected by YAP knockdown (Fig. 1D)
Response: To address this concern we performed new sets of experiments by using additional siRNAs for both mutant p53 and YAP expression. In detail, the related data for both protein and mRNA expression of cell cycle regulated genes are shown in the new right panels C and D of the new version of Figure 1 and the new Supplementary Figure 1B-C and new Supplementary Figure 2 C-E. The signal quantification for Western Blot analysis was done by Alliance 4.7 software by UVITEC (Eppendorf) as detailed in Materials and Methods.

4. *Neither p53 nor YAP knockdown was shown in SKBR3 cells (Fig. 3 A).*
Response: In the present revised version it has been included in the Supplementary Figure 2A.

5. *Almost all the IP experiments in this study used a single YAP antibody. However, the p53 pull-down band appeared to be larger than that in the extracts with a slower migration (e.g. Fig. 4A, 4C, S2D) Therefore, it is possible that YAP antibody pulled down non-specific band or it is YAP IgG cross-reaction (although IgG was used as control, it is unclear the amount and subtype of the IgG used.*

Response: To address this concern we have assessed the presence of the protein complex mutp53/YAP in additional tumor cell lines carrying mutantp53 proteins. In detail, we now show in the new Supplementary Figure 3B that mutp53/YAP protein complex is present in MDA-MB-231 breast cancer cells, FADU head and neck cancer cell line and PANC1 pancreatic cancer cells. We also performed these coprecipitation experiments with an additional anti-YAP antibody. The specific details of the used antibodies and IgGs are described in the Materials and Methods.

6. The reciprocal mutp53 IP using p53 antibody was only shown in Fig. 4B, S2A in an overexpression system. However, it is unclear whether the interaction came from GFP & mutp53 R175H interaction (Fig. 4B) or from the nonspecific false cross-reactive band (since it migrated slower than the endogenous YAP1) (Fig. S2A)?

Response: To address this concern we have performed reciprocal co-immunoprecipitation experiments in CAL27, MDA-MB-231, FADU and PANC1 cell lines using an additional antibody anti-YAP (goat polyclonal by Santa Cruz SC-1714). YAP-immunoprecipitates were probed with anti-YAP and anti-p53 antibodies (new Supplementary Figure 3C)

7. In Fig. 4E, Ig G pulled down approximately 1/3 of NF-YB (lane 2) when compared to NF-YB antibody (lane 3). If mutp53, YAP and NF-YB truly stay in the same complex as authors suggested, it is hard to believe that none of YAP and p53 were detected in IgG lane (lane 2) [it should have been 1/3 of that in NF-YB lane (lane 3), right?] (Fig. 4E).
Response: To further consolidate the findings related to the existence of the triple YAP/mutp53/NFY protein complex, we have performed in MDA-MB-468 cells immunoprecipitations using anti-YAP, anti-p53 and anti-NFY antibodies. Each of the immunoprecipitates was probed with anti-YAP, anti-p53 and anti-NFY antibodies (new Figure 4F).

8. Given the importance of the oncogenic YAP1, experiments in Fig. 5A-D with siYAP did not provide any useful information to support authors' hypothesis; In addition, siRNA effect in Fig. 5E is questionable. Since si-p53 in Fig. S4D did not show any effect on p53 expression (compare lane 4 to lane 1, Fig. S4D). In addition, if authors' hypothesis is correct that levels of YAP and mutp53 could determine the cell proliferation, the proliferation rate shown in Fig. 5E should have been YAP >YAP+sip53 >siGFP >sip53, because this is the exact order of YAP & mutp53 expression levels as shown in Fig. S4D, in which YAP & mutp53 expression levels are: YAP (lane 3)>YAP+sip53 (lane 4) >siGFP (lane 1, Fig. S4D)>sip53 (lane 2).

Response: To address this concern we have repeated the cell viability assay in epistatic conditions (new Figure 5C and new Supplementary Figure 5F). We have also evaluated cell viability by using a more a quantitative assay (ATPlite) (new Figure 5C). From these data YAP emerges as a co-factor of gain of function mutant p53 proteins which at least under these experimental condition plays a major in driving aberrant transcriptional activation of cell cycle regulated genes leading to enhanced proliferation.

9. *The specificity of drugs cerivastin and ZA is questionable. e. g. they may affect cyclin A, B, CDK1 and even p53 expression directly, which may has nothing to do with YAP, or vice versa.*

Response: We agree with the reviewer. To address this point, that has been raised also by referee 1, we performed a new experiment. We performed new experiments by reintroducing siRNA resistant YAP mutant insensitive to statin treatment (YAP-5S (Sorrentino et al. 2014) in MDA-MB-231 depleted of endogenous YAP. As shown in new Supplementary Figure 6B, statin treatment failed to reduce the expression of cyclin A, cyclin B and Cdc25c in cell expressing the non-phosphorylatable YAP mutant, meaning that the effect of statin on cell cycle genes expression relies on its ability to inhibit YAP. In line with these results, as shown by Supplementary Figure 6C, reintroduction of YAP-5SA rendered MDA-MB-231 cells significantly less sensitive to statin treatment in viability assay compared to the YAP wild type expressing counterpart.

Referee #3:

The manuscript by di Agostino et al. outlines the novel finding that mutant p53 protein promotes increased oncogenic behaviour through YAP mediated activation of NF-Y on cell cycle regulated genes. This is study represents an innovative bioinformatic analysis that clearly points to overlap of both p53mut activity and YAP pathway in cancer. The work is exemplary and has appropriate controls and clear experimental reasoning, moreover this work provides crucial evidence for aggressiveness of p53mut cancers while also highlighting therapeutic angles. It is my feeling that work should be published with minimal delay. We wish to thank the reviewer for his/her positive feeling about our manuscript The only criticism is that some discussion should be devoted to three points

1) Whether the authors have evidence that there is a natural role for YAP/NF-Y in transcription that is perturbed or altered by mutant p53, or is YAP/NF-Y a consequence of oncogenic transformation?

Response: It has been previously shown that YAP exerts its co-transcriptional activity by binding several transcription factors including the TAED family members, RUNX2 and TAp73 and TAp63. The physical interaction occurs through the WW domain of YAP and the PPXY motifs of the transcription factors. The transcription factor NFY in each of its subunits does not contain any PPXY motif that could allow the binding to WW domain Type I as that of YAP. Thus, it is unlikely that YAP could bind NFY as for the other transcription factors. On this basis we believe that the formation of the complex YAP/NF-Y and its transcriptional activity is a consequence of oncogenic transformation. Indeed, we show in the new version of Figure 4G that the complex mutp53/YAP is present but cannot be recruited at the promoters of cell cycle related genes in cells depleted for NF-Y expression.

2) Mutant p53 interacts with both p63 and p73 inhibiting their respective activities, therefore are the findings reported here independent of alternative family members? For example is the p53mut-YAP interaction dependent on p63 or p73? Does p53mut adopt a p73 like structure that facilitates binding?

Response: This a very interesting question that focuses on the composition and functional hierarchy of large protein complexes involving mutant p53 proteins. While the binding of p73 and p63 to YAP is mediated by the PPXY and the WW domain respectively, that of mutant p53 to YAP appears to be PPXY independent. This might depict different molecular scenarios: a) large protein complexes involving mutantp53 NF-Y, p73 and YAP; b) protein complexes involving mutantp53, YAP and NF-Y; c) protein complexes involving p73, NF-Y, and YAP. To date there is no experimental evidence that allow excluding that all these floating complexes can be concomitantly present in mutant p53 cancer cells. Since the complex p73/NF-Y transcriptionally inhibits cell cycle related gene expression it is unlikely that these complexes can be recruited at the *cyclinB*, *cyclinA*, CDK1 promoters in mutant p53 expressing where their expression is aberrantly upregulated. Thus, it is conceivable to hypothesise that the protein complexes mutantp53,YAP and NF-Y play a major role in the aberrant transcriptional regulation of cell cycle regulated genes which leads to hyperproliferation.

3) The authors do not address pS127-YAP1 or the nuclear localisation of YAP in this context. I would expect from their findings that pS127 is low and YAP is nuclear to allow this interaction. However, as p53mut protein is found at high levels in the cytoplasm, it equally feasible that there is both high levels of pS127-YAP in the cytoplasm that could equally be bound by p53mutant AND a level of YAP in the nucleus (p53mut bound) that remains transcriptionally active. This would be interesting follow on work but the authors should at least comment in the discussion.

Response: To address this concern we reintroduced the statin-insensitive (Sorrentino et al. 2014) YAP-5SA (siRNA resistant) in MDA-MB-231 depleted of endogenous YAP and checked the expression of cell cycle genes after statin treatment. The result is shown in the new Supplementary Figure 6B and demonstrates that statin treatment fails to reduce the expression of cyclin A, cyclin B and Cdc25c in cell reconstituted with the statin-insensitive YAP mutant. In the same experimental set-up, viability assay shown in new Supplementary Figure 6C, demonstrates that Cerivastatin is significantly less efficient in reducing proliferation in YAP-5SA-expressing cells. These results prove that YAP nuclear activity plays a major role in mediating the inhibitory effect of statins on cell cycle gene expression and cell proliferation.

2nd Editorial Decision 12 August 2015

Thank you very much for submitting your revised manuscript to EMBO reports. First of all I would like to apologize for the delay in getting back to you with a decision on it. Also, since my colleague Nonia Pariente, who is the primary editor of your paper, is currently out of the office I have taken over the handling of the manuscript to avoid further unnecessary delays.

While both referees appreciate the effort you have invested in strengthening your data, they both do not feel that it is fully conclusive yet. In particular, referee 2 still has concerns about the IP data showing the interaction between p53 and YAP. To be able to make an informed and balanced decision, we discussed this issue with referee 1 and an additional advisor. Both referee 1 and the advisor agreed that this would need to be addressed before the manuscript can be published. Our advisor suggested knocking down endogenous mutant p53 in several of the cell lines studied and test whether the band that referee 2 suspects to be the IgG heavy chain then disappears. The advisor also felt that the other concerns of the reviewers would need to be addressed.

Given the potential interest of your study we would like to give you the opportunity to revise the manuscript again. Please note that according to our policy, your manuscript would need to be accepted by November 7 (six months after the initial invitation to revise it) and that the final version would still need to be seen by one of the referees and/or the advisor. The requested additional experiments do not seem to be major, but if you do expect that you will not be able to meet this deadline, please do let us know. We would, of course, still be interested in publishing your study, but would need to assess its novelty again at this point.

I thank you in advance for your cooperation and apologize again for the delay in getting back to you on your manuscript.

REFEREE REPORTS

Referee #1:

The authors have made efforts to address the majority of my concerns and thus, the study is improved.

The Co-IP data in Fig. 4C is rather surprising. For example, the data from T47D cells indicates that almost 100% of endogenous p53 was co-immunoprecipated with endogenous YAP. Was the amount of total extract loading for p53 Western blot different from the loading for YAP western blotting? It is worth noting that p53 was not identified as a YAP interacting proteins by several high quality Hippo pathway proteomic studies.

Referee #2:

This manuscript has provided some informative insights into possible interaction between mutp53 GOF and Hippo pathway through bioinformatic assays. After revised, however the key hypothesis that YAP, mutp53, and NFY for a complex regulating cyclin B, A and CDK1 expression is still not fully supported by the evidence provided in the manuscript.

Specific comments:
1. In this study, IP is one of the most important evidence to support the hypothesis. However, the results provided did not demonstrate that a clear interaction between mutp53 and YAP. As pointed out previously, almost all IP assays using YAP antibodies in this study pulled down a "p53 band " that has a obvious larger size than endogenous p53 [e.g. Fig. 4C (T47D, SW480, SKBR3), Fig. 4F , Fig 4G, Fig. S3C, S3CD (Fadu, PANC1, MB-231)}. Given that p53 has a close molecular weight to IgG heavy chain, the most reasonable explanations is that the pull-down bands in IP assays actually represented a non-specific binding (e.g. IgG) that is not p53 (e.g. see Fig 4G). All these results again demonstrated the complexity and trickiness of the specificity of p53 IP. Especially when all the deletion mutants bound to YAP, the specificity and stringency of the IP need to be further determined.

2. Authors insisted that mutant 53 has already a basal regulation of cyclin B, A and CDK1 gene transcription. If it is also true for authors' original hypothesis as previously reported, we expect a great role of mutap53-YAP-NFY signaling after treatment by DNA-damaging agent. e.g. How about the proposed complex formation before and after DNA-damaging agent treatment (e.g ADR, 24 h)?
3. Use MCF7 cells with wild-type p53 to compare other mutp53 cell lines with different genetic backgrounds did not any help to address the specificity of Cerivastatin (despite that the reviewers had previously suggested to use isogenic cell lines).

4. All the results in this study were based on siRNA transient transfection assay. Given many uncertainties and variations of the technique, the reliability and interpretation need to be further scrutinized. For instance, In Fig. 5A, siGFP led to 1.6-fold (from 5,000 to >8,000) cell proliferation, whereas the same siGFP led to a 2.4-fold (from 5,000 to 14,000) in Fig. 5B. Therefore, it is hard to interpret the results since other siRNA's effects (siYAP, and sip53) were also within the same variation range.

2nd Revision - authors' response 25 September 2015

Referee #1:
The authors have made efforts to address the majority of my concerns and thus, the study is improved.

The Co-IP data in Fig. 4C is rather surprising. For example, the data from T47D cells indicates that almost 100% of endogenous p53 was co-immunoprecipated with endogenous YAP. Was the amount of total extract loading for p53 Western blot different from the loading for YAP western blotting? It is worth noting that p53 was not identified as a YAP interacting proteins by several high quality Hippo pathway proteomic studies.

Response: To address this specific concern we performed Co-IP experiments in the following cancer cell lines Cal27, MDA-MB-231 and SW480 that carry endogenously different human p53 mutants. Co-IP experiments were performed comparing cell lines depleted of mutant p53 protein using specific siRNAs with those expressing mutant p53 and transduced with control siRNAs. We found that the intensity of the coprecipitated mutant p53 band is strongly reduced or abolished accordingly to the efficiency of the depletion as shown by WB of the total cell lysates (new Suppl. Figure 3D). These findings further confirm the specificity of the protein complex involving mutant p53 and YAP in cancer cell lines.

2. Authors insisted that mutant 53 has already a basal regulation of cyclin B, A and CDK1 gene transcription. If it is also true for authors' original hypothesis as previously reported, we expect a great role of mutap53-YAP-NFY signaling after treatment by DNA-damaging agent. e.g. How about the proposed complex formation before and after DNA-damaging agent treatment (e.g ADR, 24 h)?

Response: Our current manuscript aims primarily to investigate the role of the transcriptional cross-talk mutant p53 and YAP in the context of aberrant cell proliferation of diverse types of human cancer cells carrying endogenously different human tumor derived p53 mutants. We are currently working on deciphering the role of mutantp53/YAP protein complex in the response to diverse anticancer agents including DNA damaging agents. These data will be the subject of a future work.

3. Use MCF7 cells with wild-type p53 to compare other mutp53 cell lines with different genetic backgrounds did not any help to address the specificity of Cerivastatin (despite that the reviewers had previously suggested to use isogenic cell lines).

Response: To address the concern raised by reviewer 2 we have performed new experiments using MDA-MB-231(mut p53 K280) cells silenced for endogenous mutant p53 and reconstituted with siRNA resistant constructs for wt p53 or mutant p53 (p53K280). Cells were exposed to cerivastatin and analysed for cell viability. As shown in the new Suppl. Figure 6G, the effect of statin is stronger in mutant p53 expressing cells.

The experiment with MCF7 cell line was performed to address the question ("*What is the effect of cerivastatin on cell proliferation and expression of cell cycle genes in wt-p53 cells and p53 null cells (preferably isogenic cell lines?")* raised by reviewer 1 about the effect of statins in wt p53 and KO p53 cell lines. Therefore in the previous rebuttal, we used MCF7 tumor cells (which harbour wt p53) silenced for p53 and treated with statin.

4. All the results in this study were based on siRNA transient transfection assay. Given many uncertainties and variations of the technique, the reliability and interpretation need to be further scrutinized. For instance, In Fig. 5A, siGFP led to 1.6-fold (from 5,000 to >8,000) cell proliferation, whereas the same siGFP led to a 2.4-fold (from 5,000 to 14,000) in Fig. 5B. Therefore, it is hard to interpret the results since other siRNA's effects (siYAP, and sip53) were also within the same variation range.

Response: To address this specific comment we now provide evidence in which we performed an additional cell proliferation assay with the related WB analysis. Either si-p53 or si-YAP caused a significant decrease of the cell proliferation when compared to si-GFP control cells. Since this new set of experiments confirmed what has been already shown in Fig. 5A-B we di not include it in the manuscript but wish to share with the reviewers.

3rd Editorial Decision 15 October 2015

Thank you for the submission of your revised manuscript. We have now received the enclosed report from the referee that was asked to assess it. We will come to a decision on your manuscript within the next 24 hours, but before we can do so, we would like to clarify two key issues, which were raised in the referee reports throughout the review process.

1. IP efficiency.
As referee #1 has pointed out in the previous report, the IP efficiency in e.g. Fig 4C, but also in other experiments seems to be very high. It appears as if 100% of the protein from the extract were pulled down. You have specified in your point-by-point response that you used 40 ug of total extract for the Western blot. Could you please provide more details concerning your protocol and in particular how much of each sample (extract, IP) was loaded on the gel. The Supplementary Materials and Methods also contain no information concerning this issue.

2. Molecular weight of p53 in the IP

As referee #2 pointed out, the band detected with the p53 antibody in the IP lane of the Western blot runs at a higher molecular weight than the band in the extract in several figures. You have done additional Co-IPs after knock-down of p53 to prove the specificity of the antibody and also used a second anti-YAP antibody, as requested. But could you please comment on the observed size discrepancy?

Referee #2

Thanks authors for providing Fig S3D to address reviewer's concern. The strength of this paper is that a large body of bioinformative work identified the interaction of mutant p53 and YAP transcriptional program. However, given authors of this paper previously reported that mutp53 promotes YAP nuclear location and YAP through metabolic pathway (Sorrentino et al., Nat Cell Biol. 2014 Apr; 16 (4):357-66.), the finding in this paper of the correlation of mutp53 and YAP activity may not be surprising, which may just merely further confirmed and are consistent with authors' previous publication (Nat Cell Biol. 2014 Apr; 16 (4):357-66). In this paper, authors proposed an additional hypothesis that based on the direct transcription interaction of mutp53, YAP and NF-Y. However, as we previous pointed out, in authors' poorly controlled IPs, YAP antibody consistently pull-down a "band larger than endogenous mutp53" which was recognized as "mutp53" by authors (Fig 4C, 4F, 4G, S3C-E). In this latest rebuttal, authors provided a new siRNA-IP (Fig S3D) to address reviewer's concern. Unfortunately, not only wasn't this new experiment well-designed, but also further validated reviewer's concern that pulled-down band may not be mutp53 but IgG or other non-specific bands recognized by YAP. Moreover, authors seem reluctant to provide evidence as suggested to show how this interaction change after DNA damage to further validate the specificity of the interaction. Finally, almost all data of this study were based on transient transfection, and artificial promoter assays, etc. No evidence that YAP overexpression indeed up-regulates endogenous Cyclin A, B, and CDK1 expression was provided.

Specific comments:
1. In all IP experiments, IgG lanes looks very clean, it is unclear whether the same amounts of IgG controls were added when compared to YAP antibody?
2. Fig S3D: Authors tried to use p53 siRNA strategy to validate the YAP IP specificity. However, the correct way to do this is to use YAP siRNA but not p53 siRNA to rule out the possibility that YAP antibody indeed does not cross react with p53 or other unknown proteins;
3. Most importantly, in this newly-provided results (Fig S3D), neither above can explain why the bands pulled-down by YAP antibody, as we pointed out before, once again are larger than endogenous mutp53?
Therefore, it is still premature to come to authors' conclusion.

3rd Revision – author's response 16 October 2015

1. IP efficiency.
As referee #1 has pointed out in the previous report, the IP efficiency in e.g. Fig. 4C, but also in other experiments seems to be very high. It appears as if 100% of the protein from the extract were pulled down. You have specified in your point-by-point response that you used 40 ug of total extract for the Western blot. Could you please provide more details concerning your protocol and in particular how much of each sample (extract, IP) was loaded on the gel. The Supplementary Materials and Methods also contain no information concerning this issue.

Response: 40 mg of protein total cell extract (4% of the protein total cell extract used for the IP) were loaded for the Western Blot analysis (Fig. 4C). 1000 mg of protein total cell extract (were either immunoprecipitated with 1ug of anti-p53, or with 1ug of anti-YAP antibodies or 1ug of purified IgGs. Since anti-p53 Ab is a sheep polyclonal antibody (Ab7, Millipore) we used the related purified sheep IgG, while for anti-YAP antibody (H-125, Santa Cruz) which is a rabbit polyclonal antibody we used the related purified rabbit IgG. As detailed in Supplementary Extensive Material and Methods we washed the IPs three times in lysis buffer and eluted them in 50 ul of SDS sample

buffer. Then, 25 ml (50% of the total IP which represent 500 mg of protein total cell extract) out of 50 ml of total IP were loaded on running gel. The related blot was probed either with anti-p53 Ab or with anti-YAP Ab. This experimental procedure was applied for all the co-IPs presented within the manuscript.

2. Molecular weight of p53 in the IP
As referee #2 pointed out, the band detected with the p53 antibody in the IP lane of the Western blot runs at a higher molecular weight than the band in the extract in several figures. You have done additional Co-IPs after knock-down of p53 to prove the specificity of the antibody and also used a second anti-YAP antibody, as requested. But could you please comment on the observed size discrepancy?

Response: Problem with Co-IP experiments is likely due to heavy chain (55 kDa) of the IP antibody used, which are also eluted from the beads and size-fractionated by SDS-PAGE together with the antigen. Since p53 (53kDa) and the antibody heavy chain are comparable in size, the migration of the antigen can be affected by the mass of the heavy chain, particularly when IgG are not crosslinked to the beads, leading to apparent increase of p53 size. This artifact is particularly clear in Supplementary Figure 3E.

4th Editorial Decision 20 October 2015

Thank you for submitting more information concerning the IP efficiency and the molecular weight of the mutant p53 protein in the IP. I have meanwhile discussed this issue with the external advisor and with our chief editor.

1. I think it is clear from your explanations that the assumed high IP efficiency originates from the loading of the gel for the Western blot (approximately 10 times more protein in the IP lane). Currently, this information is not provided in the manuscript. Please incorporate this information in the Materials and Methods section and also indicate the % loaded (4% input, 50 % IP) in the figure and/or figure legends for clarification.

2. Molecular weight of p53 in the IP
To clarify this, please add a comment concerning the observed difference in size and a potential explanation to the manuscript, either in the Results section or alternatively in the Discussion.

4th Revision - authors' response 21 October 2015

Editorial changes were made.

5th Editorial Decision 27 October 2015

Thank you for the submission of your revised manuscript to EMBO reports and for the incorporation of the requested changes. Before we can proceed with the official acceptance of your study, there are a few things that we need from the editorial side.

- Your study will be published in article format. As such, all of the Materials and Methods must be included in the main text and cannot be part of the Appendix.

- Regarding data quantification, can you please specify the test used to calculate p-values in the respective figure legends? This information is currently incomplete (e.g. in Figure legend 2) and must be provided in the figure legends.

- You have 7 figures, 6 Supplementary figures and 5 Supplementary tables. Some of your figures are currently provided as two separate files (1A, 1B, 4A, 4B, S6A, S6B). Please merge these figures into a single TIF file.
- Tables S1 - S3 appear to be data sets. If you agree that we publish these as data sets instead as Supplementary tables, please let us know and we will change it for you. Table S4 and S5 can be part

of the Appendix (see below).

- The Expanded View format allows you to highlight up to 5 Supplementary figures, which will be displayed in the main HTML of the paper in a collapsible format. If you choose 5 images as Expanded View please follow the nomenclature Figure EV1 etc. The figure legend for these should be included in the main manuscript document file in a section called Expanded View Figure Legends after the main Figure Legends section.

- For figures that are not promoted to the Expanded View, please label the file Appendix instead of Supplementary information. The Appendix includes a table of content on the first page, all figures and their legends. Please follow the nomenclature Appendix Figure S1 throughout the text and also relabel the figures according to this nomenclature.

- We now strongly encourage the publication of original source data with the aim of making primary data more accessible and transparent to the reader. The source data will be published in a separate source data file online along with the accepted manuscript and will be linked to the relevant figure.
- Please submit the source data (scanned images of blots) of your Co-IP experiments in Figures 4 and S3 together with the revised manuscript. Please include size markers for scans of entire gels, label the scans with figure and panel number, and send one PDF file per figure or per figure panel.

- Every EMBO reports paper now includes a 'Synopsis' to further enhance its discoverability. Synopses are displayed on the html version and they are freely accessible to all readers. The synopsis includes a short standfirst text as well as 2-4 one sentence bullet points that summarize the paper. These should be complementary to the abstract -i.e. not repeat the same text. This is a good place to be more informative and include, as appropriate, key acronyms and quantitative and organism (yeast, mammalian cells, etc) information. Could you please supply the bullet points, standfirst text and synopsis image?

- As part of the EMBO publication's Transparent Editorial Process, EMBO reports publishes online a Review Process File to accompany accepted manuscripts. This File will be published in conjunction with your paper and will include the referee reports, your point-by-point response and all pertinent correspondence relating to the manuscript.

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We look forward to seeing a final version of your manuscript as soon as possible. Please do not hesitate to contact me if you have further questions.

5th Revision - authors' response 29 October 2015

Editorial changes were made.

Accepted 19 November 2015

I am very pleased to accept your manuscript for publication in the next available issue of EMBO reports. Thank you for your contribution to our journal.

